Dextrose: 20.0 gm.
Distilled water, q.s: 1,000.0 ml.
pH 5.6 to 5.7 after sterilization.

(14)–(18) [Reserved]

(19) *Medium 19.*

Peptone: 9.4 gm.
Yeast extract: 4.7 gm.
Beef extract: 2.4 gm.
Sodium chloride: 10.0 gm.
Dextrose: 10.0 gm.
Agar: 23.5 gm.
Distilled water, q.s: 1,000.0 ml.
pH 6.0 to 6.2 after sterilization.

(20)–(31) [Reserved]

(32) *Medium 32.* Prepare as medium 1, except add 300 milligrams of hydrated manganese sulfate ($MnSO_4 \cdot H_2O$) to each liter of medium.

(33) *Medium 33.* Use medium 1, sterilized and cooled to 50° C. Aseptically add sufficient sterile sodium novobiocin solution to give a final concentration of 10 micrograms of novobiocin activity per milliliter of medium. Sterile sodium novobiocin solution is prepared by filtering a solution containing 2.5 milligrams of novobiocin per milliliter of distilled water through a membrane filter of 0.22–micron porosity.

(34) *Medium 34.*

Glycerol: 10.0 gm.
Peptone: 10.0 gm.
Beef extract: 10.0 gm.
Sodium chloride: 3.0 gm.
Distilled water, q.s.: 1,000.0 ml.
pH 7.0 after sterilization.

(35) *Medium 35.* Same as medium 34, except add 17.0 grams of agar to each liter of medium.

(36) *Medium 36.*

Pancreatic digest of casein	15.0 gm.
Papaic digest of soybean	5.0 gm.
Sodium chloride	5.0 gm.
Agar	15.0 gm.
Distilled water, q.s	1,000.0 ml.
pH 7.3 after sterilization	

(37) *Medium 37.*

Pancreatic digest of casein: 17.0 gm.
Soybean peptone: 3.0 gm.
Dextrose: 2.5 gm.
Sodium chloride: 5.0 gm.
Dipotassium phosphate: 2.5 gm.
Distilled water, q.s.: 1,000.0 ml.
pH 7.3 after sterilization.

(38) *Medium 38.*

Peptone: 15.0 gm.
Papaic digest of soybean meal: 5.0 gm.
Sodium chloride: 4.0 gm.
Sodium sulfite: 0.2 gm.
L-cystine: 0.7 gm.
Dextrose: 5.5 gm.
Agar: 15.0 gm.
Distilled water, q.s.: 1,000.0 ml.
pH 7.0 after sterilization.

[39 FR 18944, May 30, 1974, as amended at 40 FR 52004, Nov. 7, 1975; 42 FR 14092, Mar. 15, 1977; 47 FR 9396, Mar. 5, 1982; 47 FR 22514, May 25, 1982]

§ 436.103 Test organisms.

(a) *Preparation of test organism suspensions.* For each test organism listed in the following table, select the media (as listed by medium number in § 436.102(b)), incubation period of the Roux bottle, suggested dilution factor, and suggested storage period for the particular test organism and proceed by the appropriate method described in paragraph (b) of this section. Test organism letters A through K, M, and N correspond to those used in ''Outline of Details for Official Microbiological Assays of Antibiotics,'' A. Kirshbaum and B. Arret, ''Journal of Pharmaceutical Sciences,'' Vol. 56, No. 4, p. 512 (April 1967), which is incorporated by reference. Copies are available from the American Pharmaceutical Association, 2215 Constitution Ave. NW., Washington, DC 20037, or available for inspection at the Office of the Federal Register, 800 North Capitol Street, NW., suite 700, Washington, DC.

Test organisms	Method used	Medium used for the—		Incubation period of Roux bottle	Suggested dilution factor	Suggested storage period of suspensions under refrigeration
		Slants	Roux bottles			
Test organism A—*Staphylococcus aureus* (ATCC 6538P)[2].	1	1	1	24 hours	1:20	1 week.
Test organism B—*Micrococcus luteus* (ATCC 7468)[2].	1	1	1	24 hours	1:30	2 weeks.
Test organism C—*Micrococcus luteus* (ATCC 9341)[2].	1	1	1	24 hours	1:40	2 weeks.
Test organism D—*Staphyloccocus epidermidis* (ATCC 12228)[2].	1	1	1	24 hours	[1] 1:14	1 week.

Test organisms	Method used	Medium used for the—		Incubation period of Roux bottle	Suggested dilution factor	Suggested storage period of suspensions under refrigeration
		Slants	Roux bottles			
Test organism E—*Saccharomyces cerevisiae* (ATCC 9763)[2].	6	19	.....	.....	1:30	4 weeks.
	or					
	7	19	19	48 hours	1:30	4 weeks.
Test organism F—*Bordetella bronchiseptica* (ATCC 4617)[2].	1	1	1	24 hours	1:20	2 weeks.
Test organism G—*Bacillus cereus* var. *mycoides* (ATCC 11778)[2].	3	1	1	1 week	.....	6 months.
Test organism H—*Bacillus subtilis* (ATCC 6633)[2].	1	1	1	24 hours	.....	6 months.
	or					
	2	1	32	5 days	.....	6 months.
Test organism I—*Klebsiella pneumoniae* (ATCC 10031)[2].	1	1	1	24 hours	1:25	1 week.
Test organism J—*Escherichia coli* (ATCC 10536).	1	1	1	24 hours	1:20	2 weeks.
Test organism K—*Streptococcus faecium* (ATCC 10541)[2].	5	.....	.....	.....	.....	24 hours.
Test organism L—*Micrococcus luteus* (ATCC 10240)[2].	1	1	1	24 hours	1:35	4 weeks.
Test organism O—*Staphylococcus aureus,* resistant to novobiocin (ATCC 12692)[2].	1	33	33	24 hours	1:10	4 weeks.
Test organism T—*Saccharomyces cerevisiae* (ATCC 2601)[2].	7	19	19	48 hours	1:30	4 weeks.
Test organism V—*Micrococcusluteus,* resistant to dihydrostreptomycin (ATCC 10240A)[2].	1	1	1	48 hours	1:35	4 weeks.
Test organism W—*Pseudomonas aeruginosa* (ATCC 25619)[2].	1	1	1	24 hours	1:25	2 weeks.
Test organism X—*Mycobacterium smegmatis* (ATCC 607)..	8	36	.....	.....	.....	2 weeks.
Test organism Y—*Pseudomonas aeruginosa* (ATCC 29336)[2].	9	36	36	24 hours	1:50	1 week.

[1] If the antibiotic to be tested is paromomycin, the dilution factor is 1:25.
[2] Available from American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD. 20852.

(b) *Methods for preparation of test organism suspensions*—(1) *Method 1*—(i) *Preparation of suspension.* Maintain organisms on agar slants containing 10 milliliters of the appropriate medium. Incubate the slants at 32° C.–35° C. for 24 hours. Using 3 milliliters of sterile U.S.P. saline T.S., wash the growth from the agar slant onto a large agar surface, such as a Roux bottle, containing 250 milliliters of the appropriate medium. Spread the suspension of organisms over the entire surface of the Roux bottle with the aid of sterile glass beads. Incubate the Roux bottle at 32° C.–35° C. Wash the resulting growth from the agar surface with 50 milliliters of sterile U.S.P. saline T.S.

(ii) *Standardization of suspension.* Determine the dilution factor that will give a 25–percent light transmission at a wavelength of 580 millimicrons using a suitable photoelectric colorimeter and a 13–millimeter diameter test tube as an absorption cell. It may be necessary to adjust the suspension. Determine the amount of suspension to be added to each 100 milliliters of agar or nutrient broth by the use of test plates or test broth. Store the test organism suspension under refrigeration.

(2) *Method 2.* Proceed as directed in paragraph (b)(1) of this section, except in lieu of paragraph (b)(1)(ii) thereof, heat-shock and standardize the suspension as follows: Centrifuge and decant the supernatant liquid. Resuspend the sediment with 50 to 70 milliliters of sterile U.S.P. saline T.S. and heat the suspension for 30 minutes at 70° C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 milliliters of agar. Maintain the spore suspension under refrigeration.

(3) *Method 3.* Proceed as directed in paragraph (b)(1) of this section, except in lieu of paragraph (b)(1)(ii) thereof, heat-shock and standardize the suspension as follows: Heat the suspension for

30 minutes at 70° C. Wash the spore suspension three times with 25 to 50 milliliters of sterile distilled water. Resuspend the organisms in 50 to 70 milliliters of sterile distilled water and heat-shock again for 30 minutes at 70° C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 milliliters of agar. Maintain the spore suspension under refrigeration.

(4) [Reserved]

(5) *Method 5.* Maintain the test organisms in 100–milliliter quantities of nutrient broth—Medium 3 as described in § 436.102(b)(3). For the test prepare a fresh subculture by transferring a loopful of the stock culture to 100 milliliters of the same nutrient broth and incubate for 16 to 18 hours at 37° C. Store this broth culture under refrigeration.

(6) *Method 6.* Maintain the test organisms on agar slants containing 10 milliliters of the medium specified in paragraph (a) of this section. Incubate the slants at 32° C.–35° C. for 24 hours. Inoculate 100 milliliters of nutrient broth—Medium 13 as described in § 436.102(b)(13). Incubate for 16 to 18 hours at 37° C. Proceed as directed in paragraph (b)(1)(ii) of this section.

(7) *Method 7.* Proceed as directed in paragraph (b)(1) of this section, except incubate the slants at 30° C. for 24 hours and incubate the Roux bottle at 30° C. for 48 hours.

(8) *Method 8.* Maintain organisms on agar slants containing 10 milliliters of the appropriate medium and transfer to a fresh slant about once a week. Incubate the slants at 37° C for 48 hours. Using 3 milliliters of sterile U.S.P. saline T.S., wash the growth from the agar slant into a 500–milliliter Erlenmeyer flask containing 100 milliliters of medium 34, as described in § 436.102(b) (34), and 50 grams of glass beads. Agitate the culture by rotation at a speed of 130 cycles per minute and a radius of 3.5 centimeters at 27° C for 5 days. Determine the amount of suspension to be added to each 100 milliliters of agar by the use of test plates. Store the test organism suspension under refrigeration.

(9) *Method 9.* Proceed as directed in paragraph (b)(1) of this section, except incubate the slant and Roux bottle at 37° C and wash the resulting growth from the agar surface with 50 milliliters of Medium 37 as described in § 436.102(b)(37).

[39 FR 18944, May 30, 1974, as amended at 40 FR 52004, Nov. 7, 1975; 42 FR 14092, Mar. 15, 1977; 42 FR 18058, Apr. 5, 1977; 44 FR 10378, Feb. 20, 1979; 47 FR 22514, May 25, 1982; 47 FR 27552, June 25, 1982]

§ 436.104 Penicillin activity.

Use penicillin-free equipment and glassware.

(a) *Preparation of inoculated plates.* Proceed as directed in § 436.105(a), using 10 milliliters of medium 1 for the base layer. For the seed layer, use 4 milliliters of medium 4, inoculated with the amount of test organism C which gave the clearest, sharpest zones of inhibition measuring 17 to 21 millimeters in diameter when standardized as described in § 436.103(b)(1)(ii). Use the plates the same day they are prepared.

(b) *Preparation of working standard stock solutions and standard response lines solutions.* Proceed as directed for penicillin G in § 436.105(b), except dilute the working standard stock solution to a final concentration of 100 units of penicillin G per milliliter and use the following final concentrations for the standard response line: 0.005, 0.0125, 0.025, 0.050, 0.100, and 0.200 unit of penicillin G per milliliter. The 0.050 unit of penicillin G–per–milliliter solution is the reference concentration of the assay.

(c) *Sample preparation.* Dissolve 1.0 gram of the sample in sufficient distilled water to make 18 milliliters. Filter if not clear. Transfer 9.0 milliliters to a separatory funnel, and add 20 milliliters of amyl acetate. Add 1 milliliter of 10 percent potassium phosphate buffer, pH 2.5 (solution 11 as described in § 436.101), shake, allow to separate, and draw off the aqueous layer into a second separatory funnel. Check the pH of the aqueous solution with pH paper, and readjust with concentrated hydrochloric acid if the pH is three or above. Extract again with 20 milliliters or amyl acetate, discard the aqueous phase, and combine the amyl acetate extracts. Wash the extracts with 10 milliliters of 1 percent potassium phosphate buffer, pH 2.5, and discard the